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N-Cadherin–Catenin Interaction: Necessary Component of Cardiac Cell Compartmentalization during Early Vertebrate Heart Development

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During early heart development the expression pattern of N-cadherin, a calcium-dependent cell adhesion molecule, suggests its involvement in morphoregulation and the stabilization of cardiomyocyte differentiation. N-cadherin's adhesive activity is dependent upon its interaction with the intracellular catenins. An association with α-catenin and β-catenin also is believed to be involved in cell signaling. This study details the expression patterns of α-catenin, β-catenin, and γ-catenin, during definition of the cardiac cell population as distinct compartments in the anterior regions of the chick embryo between stages 5 and 9. The restriction of N-cadherin/catenin localization at stage 5+ from a uniform pattern *in vivo*, to specific cell clusters that demarcate areas where mesoderm separation is initiated, suggests that the N-cadherin/catenin complex is involved in boundary formation and in the subsequent cell sorting. The latter two processes lead to the specification and formation of the somatic and cardiac splanchnic mesoderm. N-cadherin colocalized with α- and β-catenin at the cell membrane before and during the time that its expression becomes restricted to the lateral mesoderm and continues cephalocaudad into stage 8. These proteins continue to colocalize in the myocardium of the tubular heart. Plakoglobin is not expressed in this region during stages 6–8, but is detected in the myocardium later at stage 13. The observed *in vivo* expression patterns of α-catenin, β-catenin, and plakoglobin suggest that these proteins are directly linked with the developmental regulation of cell junctions, as cardiac cells become stably committed and phenotypically differentiated to eventually form a mature myocardium. The localization of N-CAM also was analyzed during these stages to determine whether the N-cadherin–catenin localization was unique or whether other cell adhesion molecules were expressed similarly. The results indicate that the unique pattern of N-cadherin expression is not shared with N-CAM. We also show that perturbation of N-cadherin using a function perturbing N-cadherin antibody (NCD-2) inhibits normal early heart development and myogenesis in a cephalocaudad, stage-dependent manner. We propose a model whereby myocardial cell compartmentalization also defines the endocardial population. The presence of β-catenin suggests that a similar signaling pathway involving *Wnt* (*wingless*)-mediated events may function in myocardial cell compartmentalization during early vertebrate heart development, as in *Drosophila* contractile vessel development.

INTRODUCTION

Organogenesis involves a continuum of activations and inactivations of molecular and biochemical pathways that provide the requisite protein interactions leading eventually to stabilization of commitment and initiation of phenotypic differentiation of specific tissues. This is accompanied by tissue modeling into a three-dimensional structure. In the case of heart development current research has led to the identification of many regulatory factors in the cardiogenic region (Olson and Srivastava, 1996). These regulatory genes appear to be well conserved even across phyla because a number of *Drosophila* gene homologues are involved in vertebrate heart development. However, the information missing in the vertebrate embryo is the integration of these transcription factors with the developmental processes taking place *in vivo* to delineate the cardiac population as a separate compartment. An important aspect of early vertebrate heart development is that only the ventral portion of a seemingly homogeneous population of cells in the anterior

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bilateral mesoderm is involved in cardiogenesis and becomes incorporated into the heart. In order to fully understand the processes of cardiogenesis, it is important to define what specific events lead to the demarcation and separation of the cardiac cells as a separate compartment from a visibly homogeneous population of mesoderm cells within the bilateral heart-forming regions. The first visible event is the splitting of the mesoderm into dorsal and ventral layers by formation of the pericardial coelom in the heart region. Mesoderm splitting was first noted in the rabbit by Van der Stricht in 1895 (see Sabin, 1920). Van der Stricht described this process by stating that at the start there are no spaces between these two layers, but gradually there appear ''isolated clefts which flow together to make the cavity of the coelom.'' N-cadherin/β-catenin appear to be involved in demarcation of the boundary where the splitting occurs (see also Linask, 1992a,b).

The separation of the ventral and dorsal mesoderm to form a distinct epithelialized cardiac population in the chick embryo takes place in a cephalocaudad manner between approximately 19 and 29 hr of development. This results in the establishment of a community of myocardial cells joined by adherens junctions. A similar phenomenon known as the community effect is seen in skeletal myogenesis and has been shown also to involve cadherins (Gurdon *et al.,* 1993; Holt *et al.,* 1994). After epithelialization occurs in the heart-forming region, phenotypic differentiation proceeds as characterized by myofibrillogenesis (Tokuyasu and Maher, 1987a,b) and initiation of electrical activity (Kamino, 1991). When cell polarization and pericardial coelom formation is prevented by inhibiting the Na/K-ATPase, phenotypic differentiation is prevented and hearts do not develop (Linask and Gui, 1995). Similarly, as shown here, if N-cadherin's function is perturbed with the NCD-2 antibody, then in a cephalocaudad and stage-dependent manner, heart development is arrested and myofibrillogenesis is inhibited.

Cell–cell adhesion is a form of cellular communication and represents a way cells sense their environment. The interaction between specific adhesion molecules results in a specific biological response. The calcium-dependent adhesion molecule N-cadherin has been associated with the signaling cascade that defines the boundary of ventral splanchnic mesoderm to distinguish it from the dorsal mesoderm cells that will underlie the ectoderm (Linask, 1992a). This previous study suggested that N-cadherin-mediated expression and adhesion may be a factor in forming a distinct cardiac compartment. During gastrulation at stages 4 and 5, N-cadherin appears uniformly distributed on all cell surfaces in the bilateral mesoderm of the heart-forming region. As development proceeds from stages 5+ to 8, N-cadherin localization becomes restricted to foci of cell–cell associations to central areas of the mesoderm. Subsequently, it is within these clusters of N-cadherin localization that the first indications of mesoderm separation are observed (Linask, 1992a). Beginning with stage 5+ this compartmentalization process proceeds in an anterior–posterior manner leading to the epithelialization of the ventral population of cells. At this time N-cadherin becomes restricted to apical–lateral cell junctions. Epithelialization of the ventral cells in turn appears to stabilize the commitment of the cardiomyocytes and enables phenotypic differentiation to proceed (Linask *et al.,* 1992; Linask, 1992a,b; Linask and Gui, 1995). The *in vivo* three-dimensional microenvironment seems essential for the distinct, epithelial patterning of N-cadherin in the bilateral heart-forming regions because cells of precardiac mesoderm explants in culture exhibit an unrestricted, circumferential N-cadherin localization (Imanaka-Yoshida *et al.,* 1996; Linask and Lash, 1993).

In the present study we extend our understanding of N-cadherin's role in cardiogenesis by examining the expression of α-, β-, and γ-catenins during boundary formation and cardiac cell compartmentalization and by inhibiting its activity with a function-perturbing antibody NCD-2 in the embryo. Catenins associate with the cytoplasmic domain of many members of the calcium-dependent cadherin family, including N-cadherin (Ozawa *et al.,* 1989; Butz *et al.,* 1992; Knudsen and Wheelock, 1992; McCrea *et al.,* 1993; Peifer *et al.,* 1992; Wheelock and Knudsen, 1991). They show homology with previously identified proteins: α-catenin, with vinculin (Herrenknecht *et al.,* 1991); β-catenin, with armadillo, the product of a *Drosophila* segment polarity gene; and γ-catenin being the same as plakoglobin and sharing homology with β-catenin. The catenins link the cadherins to the actin cytoskeleton and are essential for full cadherin-mediated adhesion.

In addition to their role in cell–cell adhesion, both N-cadherin and β-catenin have been shown to have an intracellular signaling role in regulating transcription and cell differentiation (Larue *et al.,* 1996; Funayama *et al.,* 1995; Heasman *et al.,* 1994; McCrea *et al.,* 1993). Our evidence also suggests the direct involvement of β-catenin[2] in cardiac cell differentiation and heart development. Since β-catenin has been placed in the *Wnt*-signaling pathway, the involvement of a *Wnt*-mediated regulatory pathway is suggested in the events described here also. Recently, β-catenin has been shown to bind to LEF-1 and form a complex that is translocated to the nucleus (Molenaar *et al.,* 1996; Behrens, *et al.,* 1996). Thus, β-catenin may regulate gene expression by direct interaction with transcription factors such as LEF-1. Both N-cadherin and β-catenin can establish a molecular mechanism for the transmission of signals from cell-adhesion components or *wnt* protein to the nucleus. These latter aspects are targeted for future studies.

MATERIALS AND METHODS

Embryos

Fertile White Leghorn chick eggs were supplied by Truslow Farms (Chestertown, MD). Stages of development are according to the staging series of Hamburger and Hamilton (1951). Method of removal of embryos for analysis has been described (Linask and Lash, 1986).

[2] β-Catenin localization in boundary development in the cardiogenic regions in the chick embryo has been reported previously in abstract form (Linask *et al.,* 1992).

Fixation and Immunostaining

The blastoderms were fixed in Histochoice (Amresco, Solon, OH) and permeabilized in 100% cold methanol. The preembedding immunostaining and plastic embedding procedure in araldite has been described (Linask, 1992a; Linask and Lash, 1986). Sections were cut at 1 μm.

Antibodies

The following specific, well-characterized antibodies were used: Rabbit polyclonal antibody to chick N-cadherin and the function perturbing NCD-2 antibody to chicken N-cadherin are generously provided by Dr. M. Takeichi (Kyoto University, Kyoto, Japan); mouse monoclonal antibodies to chick α-catenin and β-catenin (Johnson *et al.*, 1993); mouse monoclonal antibody to bovine plakoglobin that cross-reacts with the chick antigen (see Knudsen and Wheelock, 1992) purchased from IBL Research Products Corp. (Cambridge, MA); rabbit polyclonal NCAM provided by Dr. S. Hoffman (University of South Carolina Medical College, Charleston, SC); rabbit polyclonal antibody to fibronectin purchased from Sigma (St. Louis, MO). The MF-20 mouse monoclonal antibody to sarcomeric myosin developed by Dr. D. A. Fischman was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa (Iowa City, IA) under Contract NO1-HD-2-3144 from the NICHD.

Whole Embryo Cultures and NCD-2 Perturbation

The detailed methodology for whole embryo cultures has been described (Linask and Lash, 1988a). Briefly, 52 stage 4–8 experimental embryos (20 control embryos) were placed with their ventral sides up on Nucleopore filters and then transferred onto small square filter paper rafts with central holes cut into them for observation and photography. These rafts were floated at the air–medium interface, as they rest against the sloping sides of wells of three-well slides. Embryos were cultured in the presence of 330 μl of 2:2:1 medium [2 Simm's balanced salt solution (SBSS):2 fetal calf serum:1 dilute F12X (for dilute F12X, see Linask and Lash (1988); for the contents of SBSS, see Simms and Sanders (1942)]. Just before the beginning of incubation, 5 μl of NCD-2 antibody was placed gently toward the sides of the heart-forming regions. The effective concentration of antibody using whole embryo cultures was determined to be 100 μg/ml after incubating embryos in a series of different concentrations of antibody. Control embryos received comparable amounts of 1:2 dilution of rat γ-globulin (Jackson ImmunoResearch, West Grove, PA). The antibody was observed to diffuse across the whole heart-forming region. During these early stages, before a cardiovascular system is formed and functional, nutrients are taken up by diffusion and thus the germ layers of the embryo are accessible to the antibody. Diffusion into the mesoderm has been confirmed by the application of a fluorecently labeled dextran of a similar molecular weight as that of IgG. The three-well slide was covered with a sterile slide, placed in a humid chamber, and transferred into a humidified incubator at 37°C for 22–24 hr. The next day the embryos were viewed under a Nikon stereomicroscope, photographed, and fixed in Histochoice (Amresco, Solon, OH) for further processing.

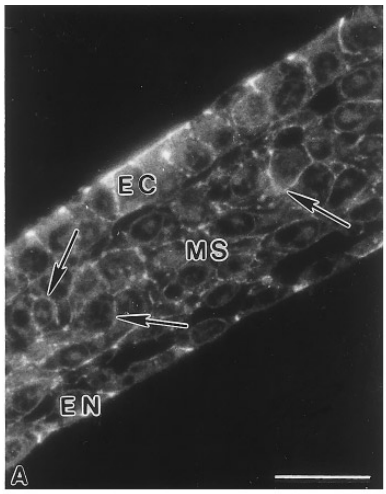
In Situ Hybridization

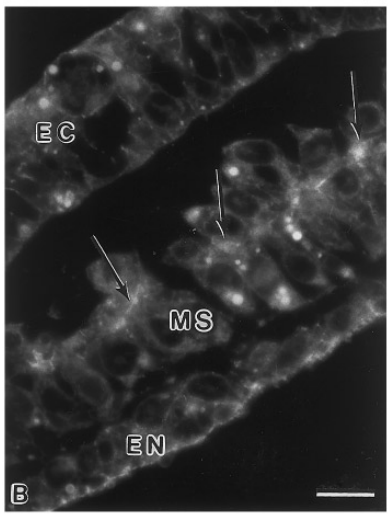
Probe. N-cadherin cDNA probe (GenBank Accession No. X07277) was kindly provided by Dr. M. Takeichi (Kyoto University, Japan) and consisted of a 3.203-kb fragment inserted into the *Eco*RI site of pBluescript (SK^+) (Stratagene, La Jolla, CA) (see Hatta *et al.*, 1988). The insert was cut to yield a 850-bp probe (between base pairs 845 to 1794) within the unique region of the gene. The 850-bp fragment was chosen as a template for the digoxigenin-labeled probes. This fragment was subcloned into Bluescript (SK^+) (Stratagene). Cloned sequences were verified by Northern analysis hybridization (see also Dalseg *et al.*, 1990) and were used to generate isoform-specific sense and antisense riboprobes for *in situ* hybridization using the Genius System kit (Boehringer Mannheim, Indianapolis, IN) and digoxigenin-UTP nonradioactive labeling. These two riboprobes were used for *in situ* hybridization experiments along with a 590-bp sense and antisense riboprobes derived from α-cardiac actin (provided by Dr. C. Emerson, University of Pennsylvania).

In situ hybridization protocol. Embryos were removed from the eggs, and the extraembryonic membranes were dissected away in cold phosphate-buffered saline (PBS). Embryos were fixed overnight at room temperature with Histochoice (Amresco), washed two times in PBT (PBS with 0.1% Tween) at room temperature, dehydrated through an ascending methanol/PBS series, and stored at −20°C. Embryos were then bleached in 6% hydrogen peroxide/methanol for 5 hr at room temperature, rinsed two times in 100% methanol, rehydrated through a descending methanol series, permeabilized with proteinase K (Sigma, 1 μg/ml) for 5 min at 37°C, and postfixed with 4% paraformaldehyde for 2 hr at room temperature. Prehybridization was performed in buffer containing 50% formamide, 5× SSC, 100 μg/ml of total yeast tRNA, 100 μg/ml denatured fish DNA, 50 μg/ml heparin, 2× Denhardt's solution for 5 hr at 63°C. The prehybridization buffer was replaced with hybridization solution (prehybridization buffer with digoxigenin-labeled riboprobe, 0.1 μg/ml) and incubated overnight at 63°C with shaking. Following hybridization, embryos were washed once with solution 1 (50% formamide, 2× SSC, 0.1% Tween) at 63°C and twice (5 and 60 min, respectively) at 63°C, once with solution 2 (2× SSC, 0.1% Tween) for 30 min at 63°C, and twice with solution 3 (100 m*M* maleic acid, 150 m*M* NaCl, pH 7.5) (30 and 60 min, respectively) at 63°C. Nonspecific binding of antibody was prevented by preblocking embryos in TBST containing 10% heat-inactivated sheep serum for 1 hr at 37°C, and by preincubating anti-digoxigenin Fab alkaline phosphatase conjugate (Boehringer Mannheim) in TBST containing 2% heat-inactivated sheep serum and 0.5% chick embryo powder made from whole 2-day-old chick embryos. After an overnight incubation at 4°C with the above preabsorbed digoxigenin antibody, the embryos were washed seven times, 1 hr each, and then incubated overnight at room temperature with TBST and 2 m*M* levamisole. The buffer was exchanged by washing twice with NTMT (100 m*M* NaCl, 100 m*M* Tris–HCl, pH 9.5, 50 m*M* $MgCl_2$, 1% Tween, 2 m*M* levamisole) for 10 min at room temperature. Antibody detection reaction was performed by incubating embryos with 0.34 mg/ml nitroblue tetrazolium and 0.18 mg/ml of X-phosphate (both obtained from Boehringer-Mannheim) in NTMT until the color reaction was complete. After the color was developed, the embryos were rinsed twice with PBT. Negative controls were performed using a sense probe. Additional controls included sense and antisense riboprobes for cardiac α-actin.

Microscopy

After whole mount observation, embryos were embedded in araldite and sectioned at 1 μm through the heart-forming regions. The





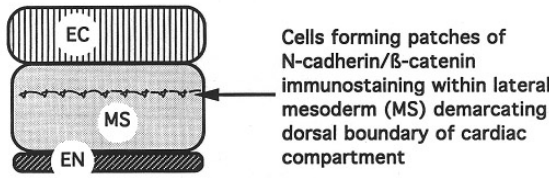


FIG. 1. β-Catenin localization using monoclonal antibody 15B8 in the lateral mesoderm of stage 5 and 5+ embryos. The embryos shown have been sectioned transversely through the bilateral heart-forming regions (HFRs). (A) The cells throughout the mesoderm (MS) of an early stage 5 embryo localize β-catenin circumferentially (arrows). Also both ectoderm and endoderm express β-catenin at cell junctions. (B) Observed at higher magnification, mesoderm cells in the bilateral HFRs at stage 5+ are beginning to show a discernible enriched localization which becomes apparent as a line in the center of the mesoderm (see arrows). Concomitantly the ventral cells begin to elongate. Some cells in a cluster with apposing boundaries showing enhanced β-catenin expression form a discernible brighter patch of staining. The patches become more evident at stage 6 (see white arrows in Fig. 2A) and demarcate the boundary where the somatic cells separate from the splanchnic cardiac cells. The diagrams show the orientation for these and subsequent figures. The circled region depicts the lateral region within the transverse section which are shown in the photographs. The lower diagram depicts the circled region in B, showing ectoderm (EC), mesoderm (MS), and endoderm (EN) and mesoderm region where patch-like N-cadherin/catenin immunostaining becomes evident. Bar in A, 20 μm; in B, 10 μm.

sections were mounted with Cytoseal 60 (Stephens Scientific) and observed with a Nikon Optiphot microscope equipped with epifluorescence. Black and white photographs were taken using Kodak Tmax ASA 400 film; color photographs were taken using Kodak Gold ASA 200 film.

RESULTS

Spatiotemporal Immunohistochemical Localization of β-Catenin, α-Catenin, and Plakoglobin during Early Heart Development

α- and β-catenins: Stages 5–8. During gastrulation at stages 4 and 5 (19–22 hr of development), mesoderm cells in the heart-forming region form a mesenchymal population of cells. By stage 7 (approximately 24 hr of development) the cells are in the process of separating into two populations, a *ventral* one consisting of epithelial myocardiocytes and endothelial (endocardial) cell precursors, and a *dorsal* one consisting of somatic mesoderm destined to line the cardiothoracic cavity. The pericardial coelom forms as a result of the described mesoderm separation. As mesoderm separation begins in the anterior regions at about stage 5+/6, small foci of cavities or clefts form in the approximate midregion in relation to the dorsal–ventral aspect of the bilateral heart-forming mesoderm. Coelom formation continues in a cephalocaudad wave across this region.

Before mesoderm separation, all mesoderm cells in the bilateral region uniformly express N-cadherin (see Linask, 1992a). Prior to mesoderm separation in the somatic and splanchnic layers, N-cadherin localizes to specific foci within the mesoderm demarcating where mesoderm separation shortly will take place (Linask, 1992a). Using a monoclonal antibody to chick β-catenin shown in Figs. 1 and 2A–2D, a similar pattern of restriction of β-catenin immunostaining as that of N-cadherin emerges (cf. Linask, 1992a). At stages 4–5 cells localize β-catenin around their circumference (Fig. 1A). Toward the end of stage 5, early stage 6, anterior mesoderm cells begin to show the restriction of β-catenin to foci within the central regions of the bilateral mesoderm (Figs. 1B and 2A). The diagram below Fig. 1B indicates the area (circled) within a cross section through the heart-forming region that is shown. Magnification of

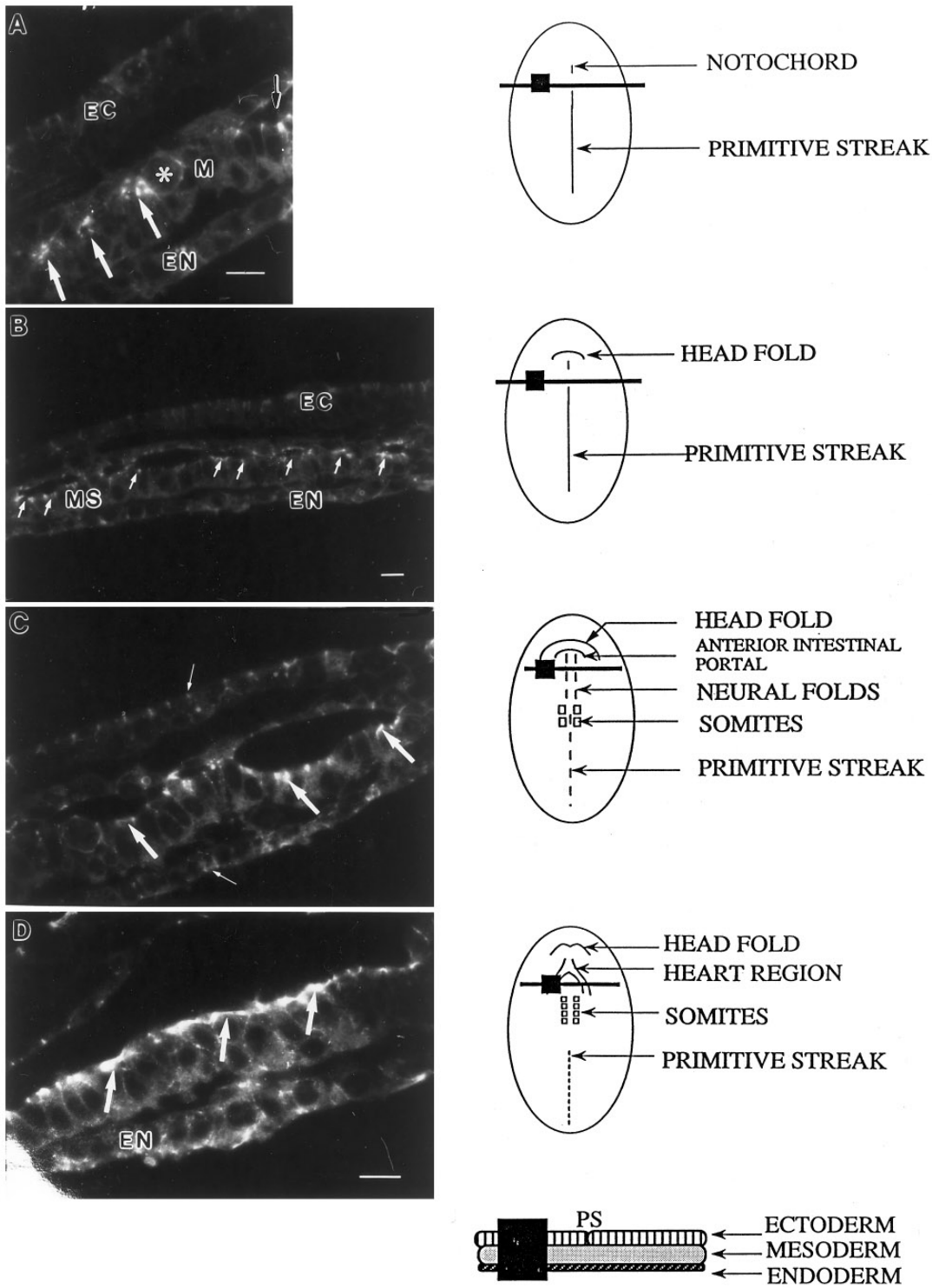


FIG. 2. Pattern of β-catenin localization between stages 5+/6 and 8 in the chick heart-forming region as detected by monoclonal antibody 15B8. Diagrams at right depict the stages of embryos analyzed, i.e., stage 6 (top) to stage 8 (4 somite pairs). The black box shows the heart-forming region from which transverse sections are obtained. Diagram at the bottom depicts the different germ layers as shown in transverse sections. (A). At stages 5+ and 6 the restriction of β-catenin continues and membrane localization is seen at apposing membranes in cell clusters (arrows) within the mesoderm. At very right of the section the somatic mesoderm and splanchnic cardiac mesoderm are already beginning to separate (small black and white arrow). (B and C) At stage 6+ to 7 β-catenin now localizes to apical lateral cell–cell junctions, as the mesoderm continues to separate into the somatic and splanchnic layers. The dorsal somatic mesoderm also shows β-catenin staining

the circled region is labeled for orientation of this and subsequent figures. This apparent restriction of β-catenin appears as a line within the mesoderm at stage 5 in Fig. 1B and is shown slightly later at stage 5+ in Fig. 2A (see white arrows). The staining for α-catenin is identical and therefore is not shown separately. All germ layers from the earliest stages express the catenins, in agreement with the ability of catenins to associate with the different cadherin family members.

As development in the bilateral heart-forming regions proceeds from stages 5+/6 to 7, expression of α- and β-catenins is seen in a star-like pattern of patches within the lateral mesoderm where the membranes of cells in a cluster meet (see arrows in Fig. 2A; small black and white arrow in Fig. 2A shows a cavity forming which is apparent in this section). This restriction of the catenin staining pattern to patch-like regions within the mesoderm coincides with the previously documented restriction of N-cadherin expression (Linask, 1992a). The catenin immunostaining patches also displayed a periodicity similar to that observed for N-cadherin. Interestingly, as previously noted also with N-cadherin, certain round cells (white asterisk), possibly undergoing mitosis and associated with the immunostaining clusters, often show staining around the whole cell.

The initial small cavities that subsequently form at sites of N-cadherin/β-catenin expression become larger, begin to coalesce with neighboring cavities, and eventually form the two amniocardiac (pericardial) vesicles. As the pericardial coelomic cavities form between stages 6 and 7+ (Figs. 2B and Fig. 2C, respectively), the catenins localize to the lateral apical regions of contact between the differentiating and elongating cardiomyocytes bordering the developing coelom (see arrows). As expected, the catenins continue to be present at cell–cell junctions in the ectoderm, somatic mesoderm, and endoderm (see small arrows in Fig. 2C). In the differentiating cardiac cells of the stage 8 embryo, the catenins remain localized predominantly to the apical region (arrows in Fig. 2D). Some more basal and lateral staining also is evident. As previously noted, in the stage 7–8 embryo, coelom formation, cardiac cell epithelialization, and differentiation proceed in an anterior to posterior progression in the heart-forming areas. By stages 7+/8 a more differentiated cardiac cell population is seen anteriorly associated with the presence of a larger coelom, in comparison to the posterior heart regions where epithelialization of precardiac cells is still ongoing.

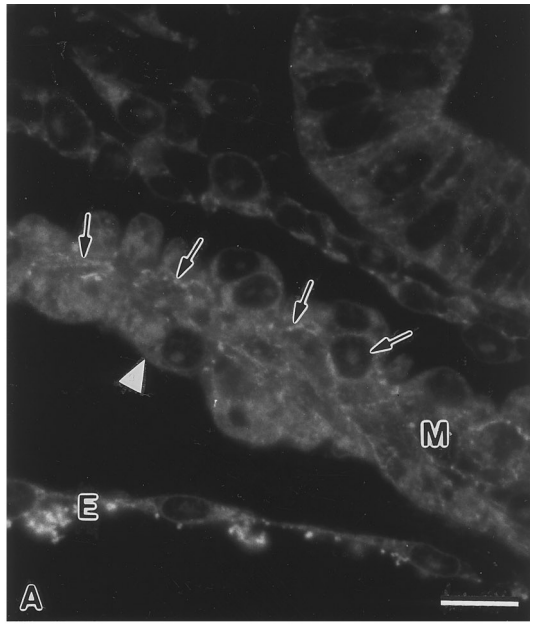
α- and β-catenins: Stages 12–13. At stages 12–13 in the myocardium of the tubular heart shown in Fig. 3, the catenins (β-catenin in Fig. 3A; α-catenin in Fig. 3B) localize primarily to adjoining cell boundaries (arrows) and are not discernible at the outer free cell surfaces facing the pericardial coelom. At this stage on the outer surface of the myocardium, the myocardiocytes appear as beads on a string. The inner most cells at the basal side of the myocardium (see white arrowheads in Figs. 3A and 3B) are associated with extracellular matrix molecules of the cardiac jelly. The catenins are found primarily at cell–cell boundaries (small black and white arrows), and are not detectable at the outer surfaces of the bead-like layer or at cell surfaces facing the basal lamina associated with the cardiac jelly (white arrowheads). Similarly, in Fig. 3C sarcomeric myosin localizes in the areas where cells are apposed. This staining (large arrow) was coinicident with N-cadherin–catenin expression at the cell contact regions and is not present at the free cell surfaces on the outer curvature or at surfaces facing the myocardial basal layer (small arrows pointing to barely visible free cell surfaces). This same myocardial immunostaining pattern is evident when the heart first begins to beat approximately at stage 10 and is not shown separately.

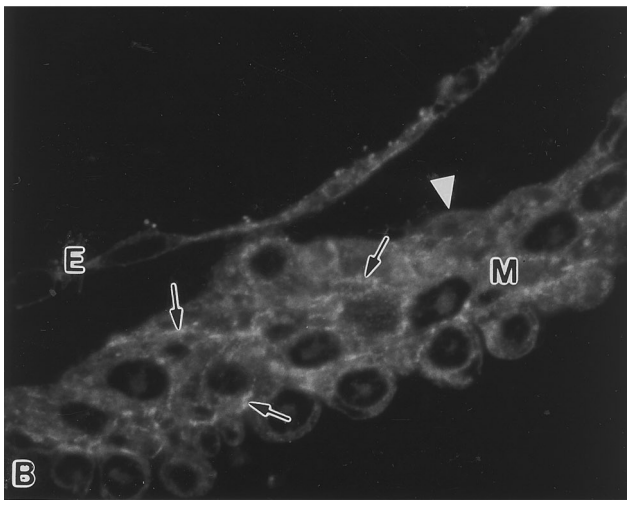
Plakoglobin. Plakoglobin, i.e., γ-catenin, shows an immunostaining pattern different from that of α- or β-catenin. Between stages 5 and 8, plakoglobin was not evident in the heart-forming regions. At stage 9–10 plakoglobin localized to apical cell junctions of a distinct cell population at the floor of the foregut just above the heart (Fig. 4, large arrows). These cells are the presumptive thyroid population. Distinct localization of plakoglobin was evident at cell junctions in the splanchnic endoderm in the extraembryonic region (small white arrow). In regions of the endocardium some plakoglobin immunostaining at cell junctions was beginning to be evident at stage 9. However, the myocardium does not express plakoglobin until later. Apparently, the expression of plakoglobin during these early stages of chick heart development is regulated independently from that of α- and β-catenin. Such developmental regulation of molecules associated with cell junctions may reflect a need to accommodate the increasing stress on myocardiocyte cell–cell interactions associated with contractility and increasing blood velocities in the embryo (Gui *et al.*, 1996). The molecular changes eventually result in mature, stable cell junctions seen in the adult tissue. Observations similar to ours were reported with respect to the later expression of plakoglobin in myocardial cell junction formation *in vitro* (Hertig *et al.*, 1996a,b).

Spatiotemporal Analysis of N-CAM between Stages 5–8

To determine whether the calcium-independent N-CAM adhesion protein also is developmentally regulated in a fash-

at its cell junctions. The pericardial coelom now becomes evident. Lower magnification of the heart-forming region is seen in B and higher magnification showing distinct immunolocalization at cell–cell junctions in C. At stage 8 in D apical localization at the membrane is observed. Some localization is also detectable at basolateral surfaces. As would be expected of cells expressing a classical cadherin(s), cells in the other germ layers continue also to express β-catenin. Bar in A, C, and D, 20 μm; in B, 40 μm. EC, ectoderm, MS, mesoderm, and EN, endoderm.





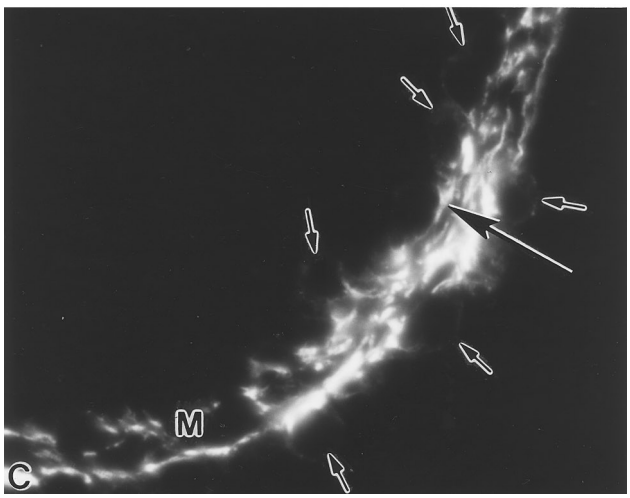


FIG. 3. At stages 12–13 the myocardium of the tubular heart is shown. The β-catenin immunolocalization is seen in A; α-catenin in B. Both catenins localize primarily to adjoining cell boundaries (arrows) and not at the outer free cell surfaces facing the pericardial coelom. At this stage the cardiomyocytes appear as beads on a string along the outer surface of the myocardium. The innermost cells at the basal side of the myocardium (arrowheads) are associated with extracellular matrix molecules of the cardiac jelly such as flectin (Tsuda *et al.,* 1996) and fibronectin (Icardo and Manasek, 1983). The catenins are found primarily at cell–cell boundaries, and are not detectable at the outer surfaces of the bead-like layer or at cell surfaces facing the basal lamina associated with the cardiac jelly. Similarly, in C sarcomeric heavy myosin immunostained with MF 20 antibody localized also to the areas where cells are apposed coinicident with N-cadherin–catenin expression (large arrow) and not at the free cell surfaces on the outer curvature or at surfaces facing the myocardial basal layer (small arrows). E, endocardium; M, myocardium. Bar, 10 μm.

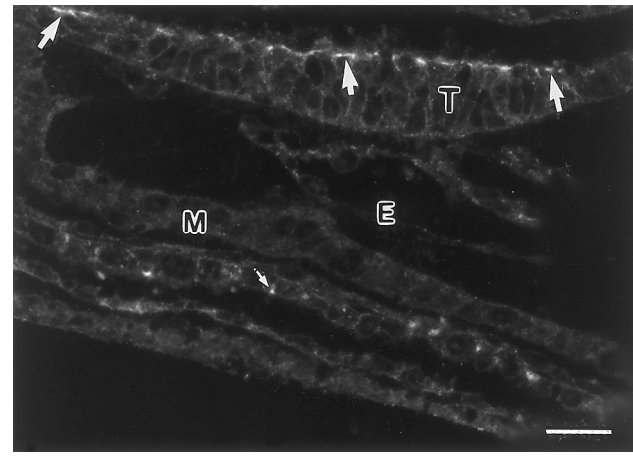


FIG. 4. Immunohistochemical localization of plakoglobin in chick heart-forming region at stages 9–10 in the chick embryo. Plakoglobin, i.e., γ-catenin, shows a different immunostaining pattern than that of α- or β-catenin and is first detectable at stages 9–10. At the earlier stages between 5 and 8, plakoglobin is not detected in the heart-forming regions. However, at stages 9–10 plakoglobin localizes to apical cell junctions of a distinct cell population at the floor of the foregut just above the heart (large white arrows) These cells are the presumptive thyroid population. Distinct localization is also evident at cell junctions in the splanchnic endoderm in the extraembryonic region (small arrow). The myocardium (M) does not yet express plakoglobin. In regions of the endocardium (E) some plakoglobin immunostaining at cell junctions begin to be evident at stage 9. Bar, 20 μm.

ion similar to N-cadherin and catenin during mesoderm sorting in the heart-forming region between stages 5 and 8, a spatiotemporal localization was carried out. We found that the pattern of expression of N-CAM did not mimic that of N-cadherin and α- and β-catenins (cf. Figs. 2A–2D). At stage 5 all of the anterior, bilateral mesoderm cells showed circumferential localization as shown in Fig. 5A. As development progresses during stages 6 and 7 (Figs. 5B and 5C,

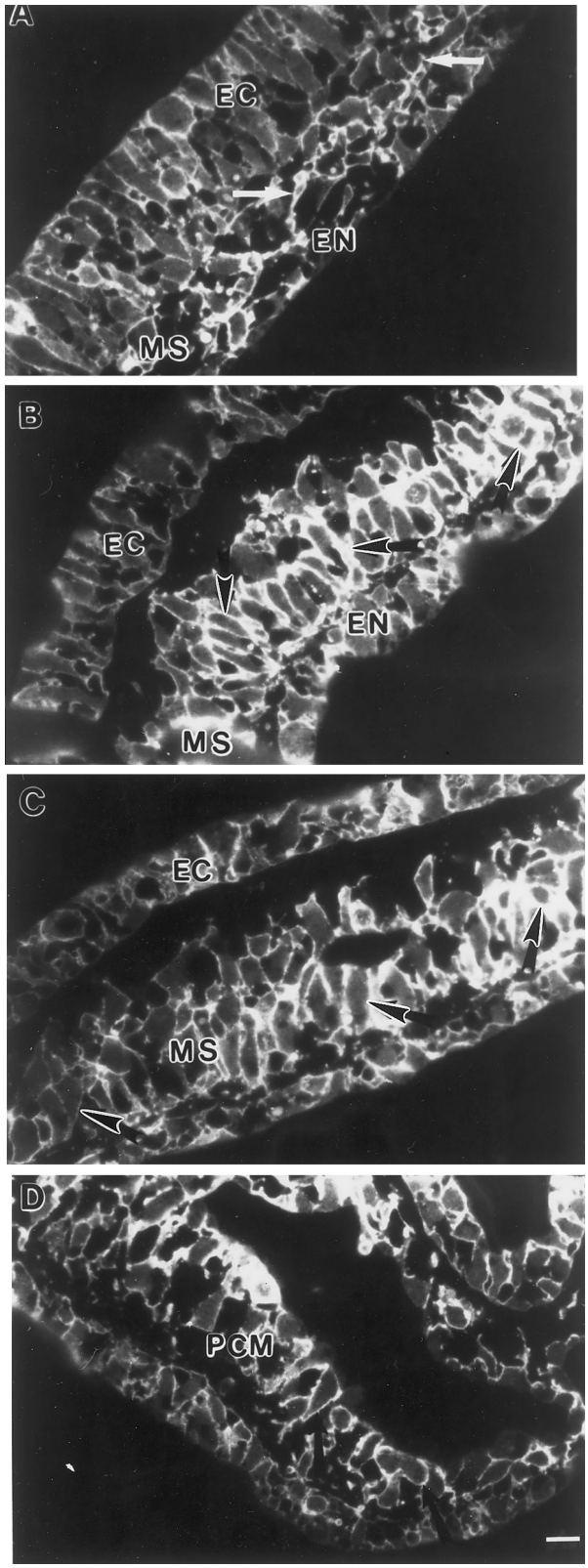


FIG. 5. The pattern of protein expression of N-CAM does not mimic that of N-cadherin/α–β-catenins during stages 5 to 8 (see A–D). For orientation of chick stages and approximate regions shown in this figure, see diagrams in Fig. 2. (A) At stage 5 all of the anteriorly bilateral mesoderm cells show circumferential localization as shown in A. As development progresses during stages 6 and 7 (B and C, respectively), the cardiac cells begin to elongate and to form an epithelium, as the coelomata form. N-CAM expression remains ubiquitous. Lateral and basolateral membrane immunostaining is specifically evident (black/white arrowheads). Cells in the heart-forming regions during stages 7 and 8 continue to show the same immunostaining pattern. Once the coelom has formed (D) the cells predominantly express N-CAM on basolateral surfaces. During these stages all cells including ectoderm, endoderm, and somatic mesoderm express N-CAM. Bar, 10 μm.

respectively) and as the cardiac cells begin to elongate, they form an epithelium. N-CAM expression was not detectable along the apical surfaces of the coelom-lining cells. However, N-CAM continued to be evident along the basolateral cell surfaces (black/white arrowheads). Cells in the heart-forming regions during stages 7 and 8 continued to show the same immunostaining pattern seen at stages 6 and 7. Once the coelom formed (Fig. 5D) the cells expressed N-CAM predominantly on basolateral surfaces. During stages 5–8 all cells, including ectoderm, endoderm, and somatic mesoderm, expressed N-CAM.

In Situ Hybridization Analysis of N-Cadherin mRNA Expression

To determine whether the previously reported restriction of N-cadherin expression to cell clusters within the mesoderm is being regulated at the transcriptional level, an *in situ* hybridization analysis of N-cadherin mRNA was carried out. Expression of N-cadherin in a stage 7+ chick embryo exposed to antisense probe is shown in Fig. 6A (purplish-brown signal) and to sense probe (control embryo) in Fig. 6B. The control embryo shows no background hybridization signal. As shown in the whole mount in Fig. 6A, N-cadherin message is present in the stage 7 chick embryo in the heart-forming regions, as well as in the developing brain, somites, Hensen's node, and neural plate. Sectioning of the heart-forming region indicated that N-cadherin message is expressed by all mesoderm cells in this region and not only in clusters of cells. Brain development in the control embryo, which like the antisense embryo shows three somite pairs, appears slightly behind in the antisense embryo by its not showing neuropore formation (Fig. 6A). Thus, the concentration of N-cadherin to specific loci occurs on a posttranslational level.

Perturbation of Early Heart Development Using the N-Cadherin Function Perturbing Antibody NCD-2

To determine whether perturbing N-cadherin-mediated cell–cell adhesion would affect early heart tube development and subsequent myofibrillogenesis, 62 chick embryos

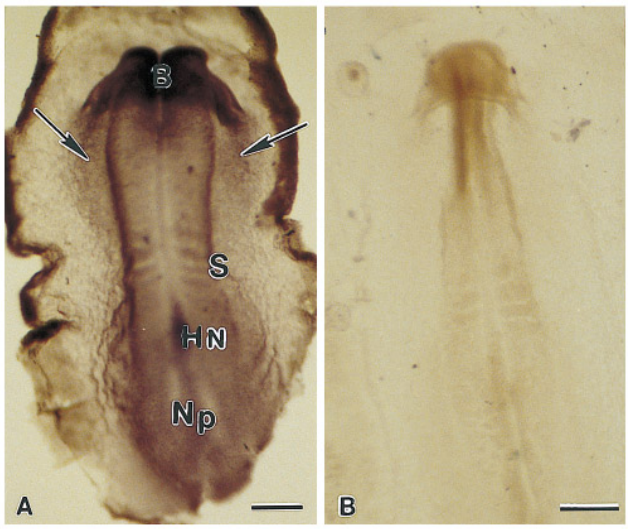


FIG. 6. *In situ* hybridization of N-cadherin mRNA at stage 7+ in the heart-forming regions of the early chick embryo. Expression of N-cadherin in embryo exposed to antisense probe is shown in A and to sense probe (control embryo) in B. Although both embryos show 3+ somites, the brain region in embryo A is slightly more advanced in its development, as can be seen by the neuropore that is developing. As shown in the whole mount in A, N-cadherin message is present in the stage 7+ chick embryo in the heart-forming regions (arrows) and in the developing brain region (B), neural plate (Np), somites (S), and Hensen's node (HN). Sectioned the heart-forming region indicated that all mesoderm cells in the bilateral anterior regions expressed N-cadherin message. Control embryos showed no hybridization signal with the sense probe. Bar in A, 375 μm; in B, 240 μm.

between stages 5 and 8 were treated with the N-cadherin function-perturbing antibody NCD-2 (Hatta *et al.*, 1985). Control embryos incubated in normal rat IgG showed no immunoglobulin-perturbing effects. In Figs. 7A and 7B a control embryo incubated at stage 5 is shown after 22 hr incubation and staining with MF20 monoclonal antibody against sarcomeric myosin. Note the presence of a normal, looping, contractile heart. At this stage only cardiac cells express sarcomeric myosin heavy chain. Embryos treated with NCD-2 antibody showed stage dependent effects after 22 hr incubation (Figs. 8–10). In all regions shown previously to express N-cadherin (neural tube, heart, and somites), normal development was perturbed by the NCD-2 antibody. A detailed analysis of effects on neurogenesis and somitogenesis will be described in separate articles.

In relation to heart development when the embryos were exposed to NCD-2 at stage 4, no hearts formed, nor was cardiac myofibrillogenesis detected, as assayed by immunostaining for sarcomeric myosin using the MF-20 monoclonal antibody (Fig. 8A). When the embryos were treated at stages 5–7, various lengths of heart tubes were present and myofibrillogenesis was evident with only the most anterior parts of the cardiac tube having MF-20 staining (Fig. 8B; also see Figs. 9C and 9D). In Figs. 9A and 9B two representative embryos are shown after incubation. The respective sarcomeric myosin immunostaining is shown in Figs. 9C and 9D to identify the cardiac cells that are undergoing phenotypic differentiation. Note that the developing brain region is perturbed by the N-cadherin-perturbing antibody and appears shortened in comparison to the brain region in control embryo. The posterior somites continue to develop and appear normal in shape. The posterior somite development indicates that the embryos remain viable during the 22-hr incubation period, even though heart and neural tube development is perturbed significantly anteriorly. However, the embryos will die with further incubation because the cardiovascular system is severely compromised. The embryo in Fig. 9A shows two tubular hearts that appear close together in the anterior part of the embryo and are situated in front of the developing brain (see arrows in Figs. 9A and 9C). This is due to abnormal extension and development of the brain region, a condition often seen in N-cadherin perturbed embryos. In Fig. 9B the embryo shown was placed in culture at stage 5. At the end of the incubation period this embryo shows distinct cardiabifida in that two small cardiac tubes detected also by MF20 antibody staining are seen to be developing on either side of the embryo in the anterior bilateral regions (shown in Fig. 9D).

To determine the extent of anterior/posterior differentiation of the heart regions, fibronectin (FN) localization was determined. FN undergoes specific expression patterns during heart development to eventually become highly organized in the cardiac jelly during looping (Icardo and Manasek, 1983; Linask and Lash, 1986). A similar embryo as shown in Figs. 9B and 9D was immunostained for fibronectin and sectioned. Cross sections through the heart-forming region of this embryo going from anterior to posterior are shown in Figs. 10A–10C. In Fig. 10A the neural tissue had not developed a tube and remained visible as a plate (NP). Anteriorly two heart tubes with cardiac jelly present were seen on either side of the embryo. Presence of cardiac jelly would be expected at this developmental time point during incubation. One such heart tube is shown in Fig. 10A. As expected, fibronectin localization is seen in the basement membranes and in the cardiac jelly of the anterior tubular heart region. In the more posterior sections, organization of the mesoderm becomes more disrupted. As shown in Fig. 10B, the ectoderm of neural region is thrown into undulating folds with fibronectin apparent in the basal lamina underneath the ectoderm (slightly out of focus). The lateral mesoderm in this region was disorganized. However, a distinct mesoderm compartment was apparent (arrow) with fibronectin localizing at the mesoderm–endoderm interface underneath the precardiac population. No further differentiation has occurred. At the posterior end of the heart-forming region (shown in Fig. 10C), the mesoderm failed to separate normally. The cardiac cells are not elongating properly, although fibronectin deposition at mesoderm–endoderm interface is apparent, as one would expect in normal embryos (see Linask and Lash, 1986).

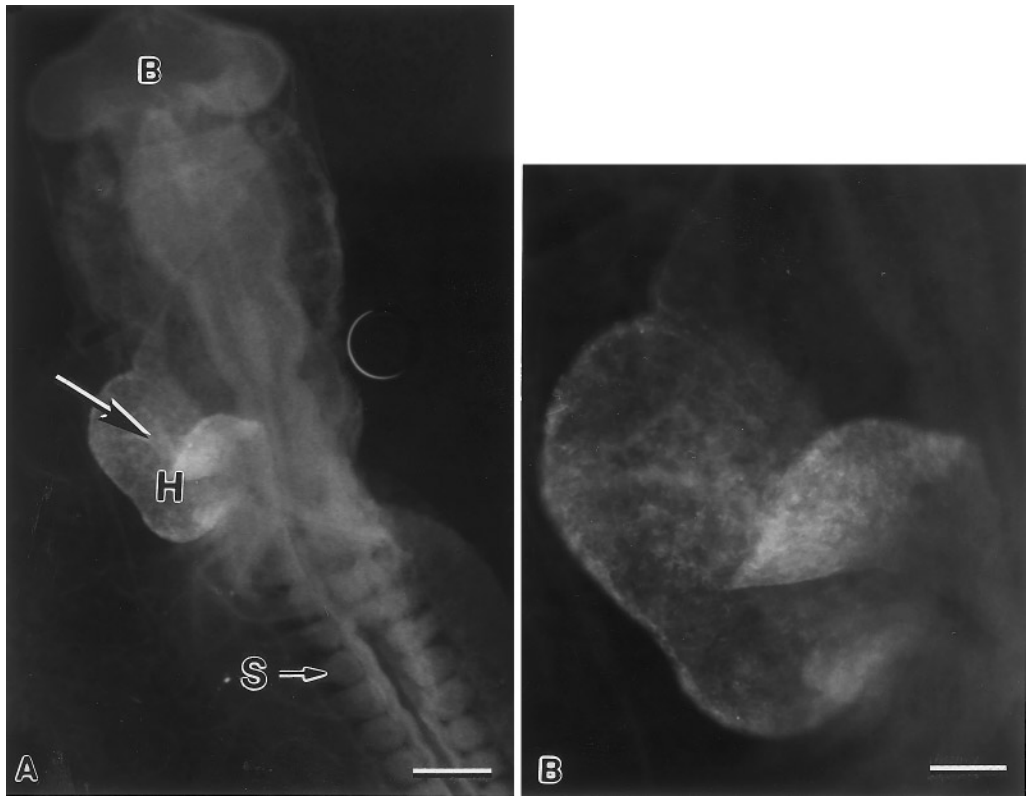


FIG. 7. Control embryo placed in culture at stage 5 and incubated in the presence of normal rat IgG for 22 hr. Embryos were fixed and immunostained for sarcomeric myosin heavy chain using MF 20 monoclonal antibody. (A) Note the extended developing brain (B) region. The heart (H) which is positive for sarcomeric myosin (arrow) is looping normally to the embryonic right. Somite pairs (S), as viewed from the ventral side of the embryo, are also normal in appearance. B is a higher magnification of the heart showing localization of sarcomeric heavy myosin. Brighter area of staining reflects the focal plane and area where the heart is beginning to fold upon itself during the looping process. Bar in A, 200 μm; in B, 100 μm.

DISCUSSION

β-Catenin and Similarities in Drosophila and Contractile Vessel Development

The state of specification of a cell *in vivo* in response to regulatory signals and the microenvironment influences protein–protein interactions and intracellular protein localization. Uniform cellular expression of the *Drosophila* homologue of β-catenin, i.e., Armadillo, is seen in the eye–antennal imaginal disc in cells that are still undifferentiated and are positioned in front of the morphogenetic furrow that passes across the eye (Peifer and Weischaus, 1990). As the furrow passes, Armadillo becomes concentrated in a star pattern on the membranes between the cells joining together to form the initial photoreceptor clusters. Actin eventually becomes enriched in the same regions, but only after Armadillo concentrated in the same region. This pattern of events is similar to the concentration of β-catenin expression at cell–cell boundaries within clusters of lateral mesoderm cells within the heart-forming region. Stabilization of β-catenin in N-cadherin-mediated adhesion, as well as in signaling, appears to depend on *Drosophila wingless*/vertebrate *Wnt* signaling (Peifer, 1995; Peifer *et al.*, 1994; see also Hinck *et al.*, 1994). Possibly in early chick heart development signaling by the *Wnt* pathway cooperates synergistically with fibroblast growth factor/bone morphogenetic protein (FGF/BMP) signaling to induce the ventral mesoderm (Ranganayakulu *et al.*, 1996; Schultheiss *et al.*, 1995; Sugi and Lough, 1995). The regulation of cadherin–catenin interactions in vertebrate heart development may be part of a pathway similar to that in *Drosophila*. Importantly, in *Drosophila*, contractile vessel (''heart'') development requires *wingless* (Park *et al.*, 1996; Wu *et al.*, 1995). Elimination of *wingless* function for a short time period after gastrulation in *Drosophila* results in the selective loss of heart precursors (Ranganayakulu *et al.*, 1996; Wu *et al.*, 1995). The analyses here show an early critical time during which N-cadherin/β-catenin function is necessary for compartmentalization of vertebrate heart precursor cells. The data are consistent with the likelihood that Wnt signaling may be involved in the regulation of the N-cadherin/β-catenin-mediated events associated with vertebrate heart development. Recently the Wnt2 gene was reported to be expressed in the early mouse

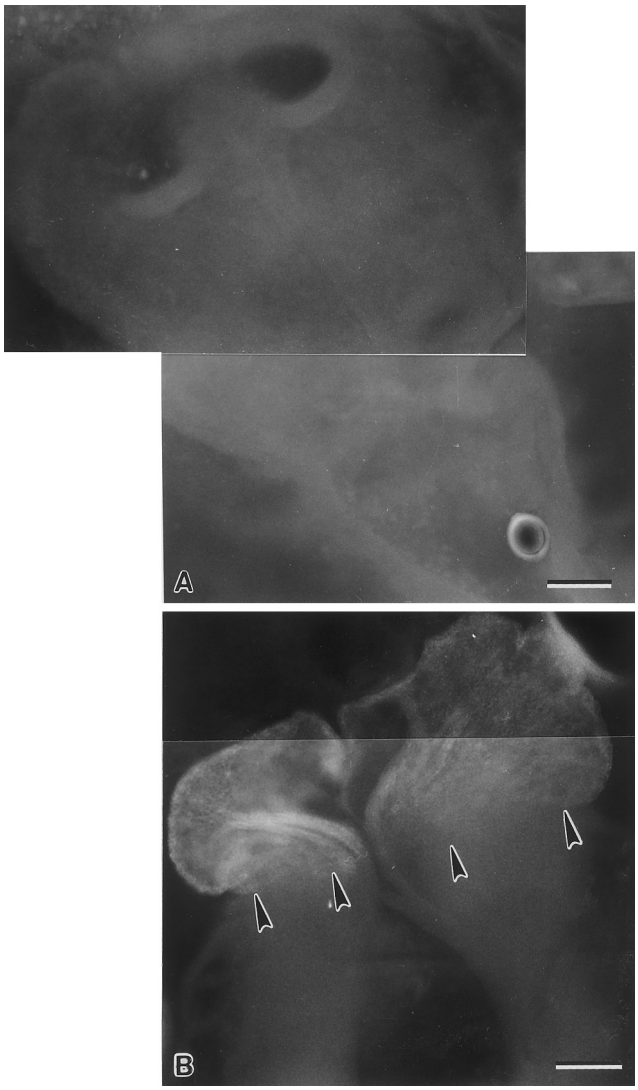


FIG. 8. Embryos treated with the NCD-2 N-cadherin-perturbing antibodies show stage-dependent effects. If the embryos were exposed to NCD-2 at stage 4 and then incubated for 20–22 hr, neither hearts nor heart tubes were discernible; nor was cardiac myofibrillogenesis evident, as detected by immunostaining for sarcomeric myosin heavy chain using MF-20 monoclonal antibody. A montage of one such embryo is shown in A. In these antibody-perturbed stage 4 embryos, the shortened neural tube and anterior somites showed abnormalities, as did the heart. (B) If the embryos were treated at stages 5–7, then various lengths of heart tubes and myofibrillogenesis were evident with only the most anterior parts of the cardiac tube showing MF-20 staining. In addition, these tubes usually were beginning to show looping. Arrowheads point to the boundary of myofibrillogenesis, after which the remainder of the tube is negative. Bar in A, 200 μm; in B, 100 μm.

heart field of 7.5–8 days of gestation (Monkely *et al.*, 1996). This coincides with the period of events described here. Whether Wnt2 is expressed and active in the chick cardiogenic crescent remains to be determined.

Epithelialization, Pericardial Coelom Formation, and Phenotypic Differentiation of Cardiac Cells

Exciting progress has been made in understanding the regulatory genes and growth factors that regulate heart development and cardiac myogenesis. Several regulatory factors have been identified (Olson and Srivastava, 1996). For example, the GATAs (Laverriere *et al.*, 1994), Nkx 2.5 (Schultheiss *et al.*, 1995), and MEF2C (Edmondson *et al.*, 1994) are expressed by the mesoderm cells in the chick cardiogenic region during early stages, as are cardiac-inducing factors being synthesized by the anterior endoderm (Dersch and Zile, 1993; Kostetskii *et al.*, 1995; Schultheiss *et al.*, 1995; Sugi and Lough, 1994, 1995). At present it is not understood how these factors relate to the early developmental processes ongoing in the embryo that set aside a distinct compartment of cardiac cells comprised of ventral mesoderm and exclude the dorsal mesoderm. Compartmentalization may result in a restriction of the heart field and apparently leads to a stabilization of commitment of cardiomyocytes and endocardiocytes, as they begin to differentiate irreversibly into phenotypic cardiac cells.

Our research suggests an important role for cell adhesion molecules, specifically for N-cadherin and catenins, during compartmentalization in cardiogenesis. N-cadherin is involved in epithelialization of the ventral cardiomyocyte population of cells, as the bilateral anterior mesoderm splits into the dorsal somatic mesoderm population and the ventral cardiac population. The pericardial cavity forms as a result of this separation. The above-mentioned regulatory factors appear to be involved in the specification of the cardiac population, but stable phenotypic differentiation of cardiac cells seems to need the establishment of stable cell junctions. The potential for cardiac cells to differentiate is present as early as the blastodisc stage. It has been reported that cells removed from the blastodisc at stages 3–4 can differentiate into cardiomyocytes *in vitro* (Antin *et al.*, 1994; Gonzalez-Sanchez and Bader, 1990; Holtzer *et al.*, 1990). Importantly, the cells do so only in clusters and not as individual cells.

Despite the necessary cellular regulatory factors being present earlier at stages 4–5, cardiac phenotypic differentiation is not seen *in vivo* until later. Phenotypic differentiation begins in anterior regions at approximately the stage 5+/6 developmental window. At this time a signaling event stimulates the cells to sort out and initiate phenotypic differentiation in a cephalo-caudad manner. *In vivo* this inductive event is accompanied by the mesenchymal cardiac population sorting out and the cells undergoing a shape change by elongating and forming tight junctions and a true epithelium. These epithelialized cells shortly begin to show cardiomyocyte characteristics, as myofibrils and electrical activity, and become irreversibly differentiated cells. The time of exposure to exogenous perturbing agents, in relation to advancement of epithelialization along the anteriorposterior axis, will determine the extent to which normal cardiomyogenesis is disturbed. The earlier the stage at which exposure to the N-cadherin perturbing antibody occurs, the

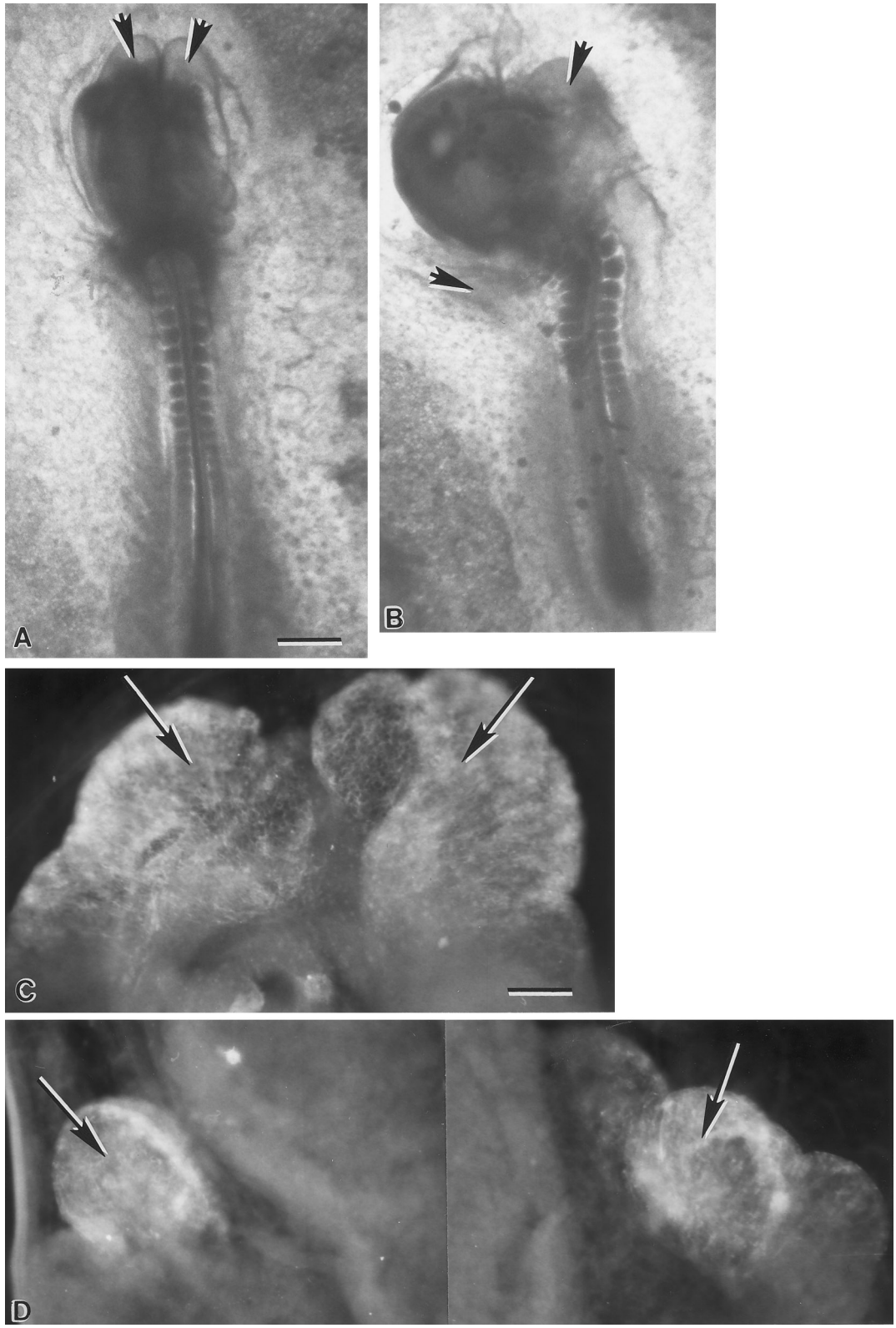
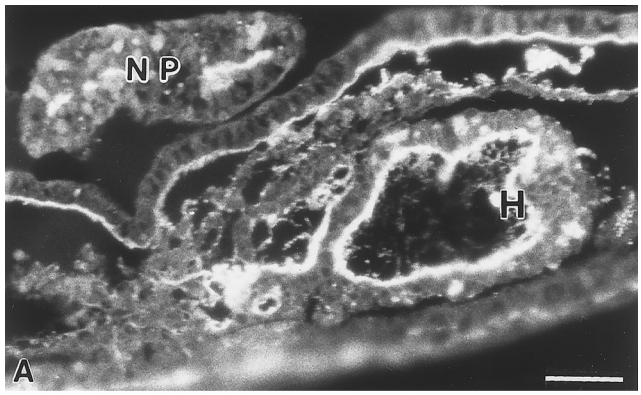
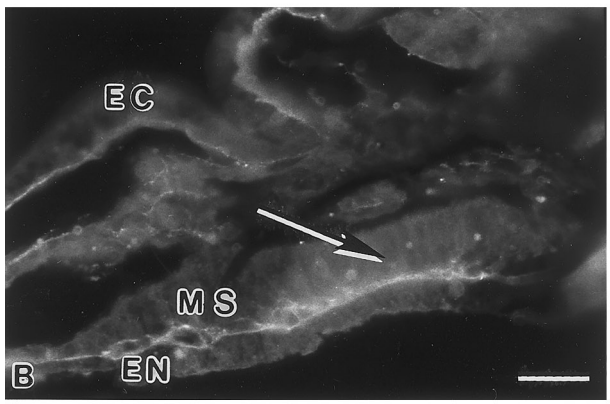


FIG. 9. Two other antibody-perturbed embryos are shown that were placed in culture and treated at stage 6+ (A and C) and stage 5 (B and D), respectively, and then incubated for 22 hr. Both embryos displayed abnormal neural tube development and a shortening of the developing brain region in comparison to the control embryo. As a result, the cardiac tubes in embryo in A and C appear to be above the developing head region (arrows). After sarcomeric myosin heavy chain localization using MF-20 antibody, only the anterior portions of the tubes express the antigen. In the embryo shown in B and D cardiabifida has resulted with two localized regions of cardiac tissue apparent by MF-20 immunostaining (see arrows). Bar in A and for B, 200 μm; in C and for D, 60 μm.





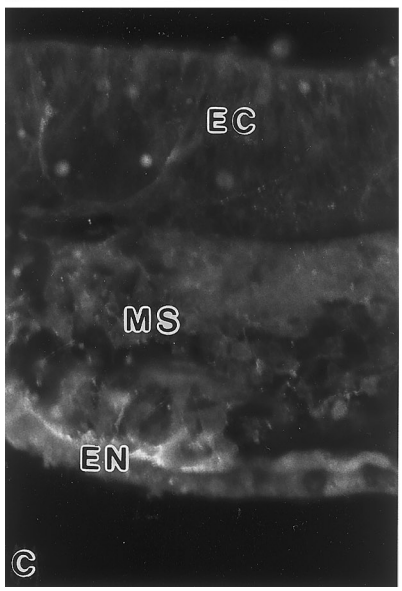


FIG. 10. Sectioning of a NCD-2 antibody-perturbed embryo similar to that shown in Fig. 9B. Various developmental anomalies were evident in the transverse sections shown anterior to posterior in the lateral developing heart areas. This embryo was stained with an antibody against fibronectin. Fibronectin expression during heart development has been previously characterized and can serve as a marker for the anterior/posterior gradient of heart development. (A) In anterior sections the neural tube has not developed normally and remains visible as a plate (NP). Cardiabifida is seen by the presence of two heart tubes with cardiac jelly present on either side of the embryo. One such heart tube (H) is shown in A. This tube expresses fibronectin at the basal side of the myocardium and within the cardiac jelly, as would be expected of a normal embryo incubated for 22–24 hr. In B more posteriorly, but still within the heart-forming region, the neural region is thrown into folds with fibronectin apparent in the basal lamina underneath the ectoderm (out of focus). The lateral mesoderm in this region is very disorganized. However, a ventral mesoderm (MS) compartment (arrow) with fibronectin localizing underneath is apparent at the mesoderm–endoderm interface. No further cardiomyocyte differentiation has occurred. At the posterior end of the heart-forming region shown in C, the mesoderm has failed to separate normally. Fibronectin is still being synthesized. EC ectoderm; MS mesoderm; EN endoderm. Bar in A, 60 μm; in B and C, 30 μm.

smaller is the anterior region expressing cardiac sarcomeric myosin. Embryos exposed at stage 4 develop neither tubular cardiac structures nor cardiac tissue. These results indicate that if one perturbs N-cadherin interactions before the mesoderm separates, tubular heart structures do not develop and myofibrillogenesis does not occur. If embryos are exposed at stage 7 and epithelialization has taken place normally in anterior regions, then cardiac cells in anterior regions can go ahead and differentiate, organize myofibrils, and form tubular structures, but posterior heart-forming regions remain undifferentiated. If the embryos are exposed at stage 8 or later, normal cardiogenesis takes place indicating that the N-cadherin-mediated events for all cardiogenic cells along the anterior/posterior axis have been completed and stabilized by this stage.

Myofibrillogenesis during cardiac myogenesis *in vitro* has been shown to involve N-cadherin (Goncharova *et al.*, 1992; Imanaka-Yoshida *et al.*, 1996; Soler and Knudsen, 1994). This also appears to be true *in vivo* for cardiac myofibrillogenesis. It is at apposing cell–cell junctions within the developing myocardium of the tubular heart that we first detect cardiac sarcomeric myosin. It has been reported by others that it is within the apical regions of cardiac cells lining the coelom of stage 7 embryos that myofibrils first organize (Han *et al.*, 1992; Tokuyasu and Maher, 1987a,b). Significantly these regions coincide with N-cadherin/catenin expression. *In vitro* analyses of myofibrillogenesis in precardiac mesoderm explants show a similar pattern in relation to N-cadherin as reported here (Imanaka-Yoshida *et al.*, 1996). If either stage 5–7 embryos or precardiac explants are exposed to N-cadherin perturbing antibodies, myofibrillogenesis is inhibited. Thus, initially the N-cadherin/β-catenin complex appears important in boundary specification and cardiac cell-shape changes *in vivo*. Subsequently, it may serve as a nucleating center for the initiation of myofibrillogenesis.

The epithelialization step of cardiomyocytes may at the same time define the endocardial cell population. We propose that endocardial cells arise from those cells in the ventral splanchnic mesoderm that initially remain mesenchymal and do not become part of the compartment joined by N-cadherin-mediated adhesion junctions at the mesoderm boundary (see Fig. 11). Endocardial cells neither epi-

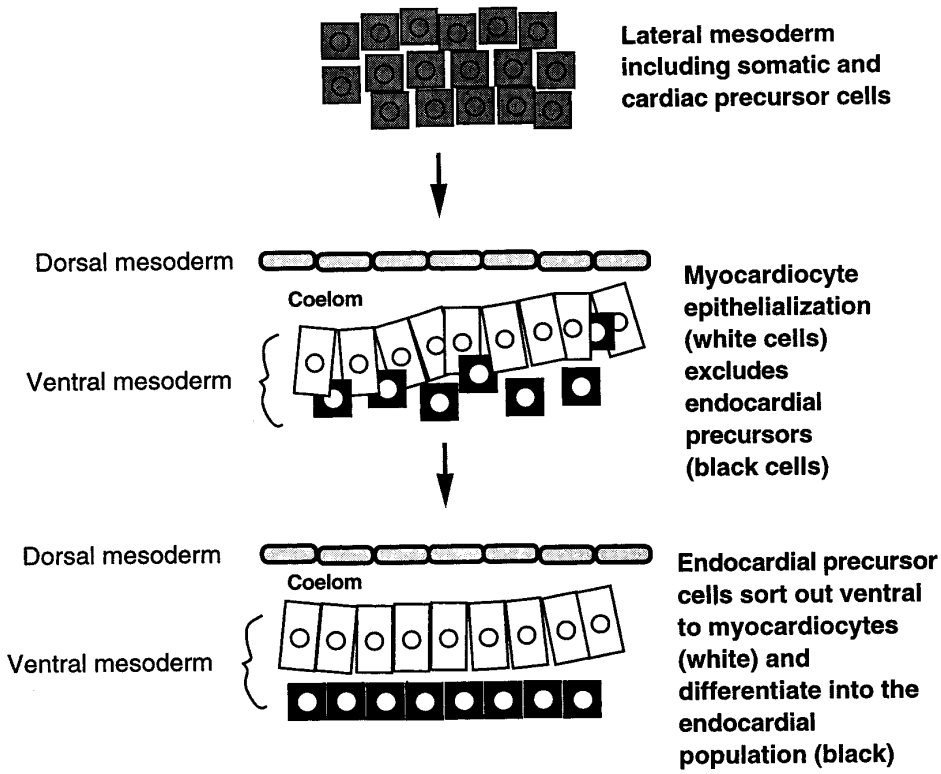


FIG. 11. Model proposed for the sorting out of the mesoderm into the somatic and splanchnic populations. Initially the lateral mesoderm (dark stippling) is a mesenchymal population of cells that includes precursors to somatic mesoderm cells and precardiac cells (both premyocardiocytes and preendocardiocytes). All of these cells express N-cadherin and β-catenin at stages 4–5. At some signal, possibly *Wnt*-mediated, boundary formation is activated, as detected by a restriction of N-cadherin/β-catenin expression to localized foci of cell–cell associations within the mesoderm. These foci serve to demarcate the ventral mesoderm from the dorsal mesoderm, as subsequently mesoderm separation begins in association with these clusters. The ventral mesoderm cells become linked together by N-cadherin-mediated adherens junctions. This results in a mesenchymal to epithelial cell shape change in this cell population establishing a differentiating myocardiocyte compartment (white cells). Epithelialization excludes a population of precursor cells that are positioned more ventrally (black cells). After cardiomyocyte compartment formation, the presumptive endocardial cells can sort out only by moving toward the endoderm and there into a permissive (or instructive) microenvironment for endothelial–endocardial cell differentiation. As they differentiate into the endocardial population, these cells down-regulate N-cadherin expression and begin to express vascular cadherin. Thus, the somatic and splanchnic precursors may be pluripotent before layer separation and cell specification may occur as a consequence of mesoderm layer separation.

thelialize nor become part of the elongating myocardiocyte compartment. The cells forming the tightly joined myocardiocyte population essentially form a dorsal barrier to any cell movement in that direction. Thus, the mesenchymal cells can only sort out ventrally toward the mesoderm–endoderm interface. The microenvironment at the mesoderm–endoderm interface is a permissive and possibly an instructive environment for endothelial–endocardial cell differentiation. A number of molecules that have been shown to stimulate endothelial cell differentiation and vasculogenesis such as fibronectin (Linask and Lash, 1986; Risau and Lemmon, 1988) and growth factors, including FGF and members of the TGF-β family, are secreted by the endoderm (Sugi and Lough, 1995). Supporting our hypothesis, we have never observed in quail embryos expression of the quail-specific endothelial marker, QH-1, within the epithelial population of cells (unpublished observations). This appears to be similar to the observations on quail embryos of Sugi and Markwald (1996). Moreover, only the cardiomyocyte population in avian embryos maintains N-cadherin expression. The endocardial cells initially express N-cadherin, but apparently begin expressing a vascular cadherin and sort out from the cardiomyocyte population (Lampugnani *et al.*, 1992; see also Linask and Lash, 1993). Because N-cadherin appears to be involved in initiating myofibrillogenesis, this may explain why some endocardial cells initially organize myofibrils (see Tokuyasu *et al.*, 1987a), before they completely down-regulate N-cadherin and sort out. Thus, the heart-forming mesoderm may be considered bipotential (Linask and Lash, 1993; Cohen-Gould and Mikawa, 1996). Which precardiac cells form the myocardial and which the endocardial endothelial cell types could be determined by

their position relative to the epithelialization process subsequent to boundary formation.

The molecules that are involved in demarcating where the mesoderm separates thus appear as important regulatory and possibly signaling molecules for subsequent phenotypic cardiomyocyte differentiation. We propose that the restricted expression of β-catenin and the association of β-catenin/α-catenin in complexes with N-cadherin demarcate the boundary of cardiac mesoderm and serve to initiate compartmentalization of the cardiomyocyte population. The adhesion appears to spread from one N-cadherin/catenin-expressing cluster of cells to another. This process establishes a community of cells and sets up the requisite protein interactions to act as a nucleating center for subsequent myofibrillogenesis. There appears to be a hierarchy of regulation. The formation of the initial clusters seems not to be affected by N-cadherin-perturbing antibody treatment, suggesting a cadherin-independent molecule or another cadherin(s) may be important in the clustering event. However, the spreading of adhesion from one cluster to another appears to be most susceptible to N-cadherin antibody perturbation and results in the inhibition of mesoderm separation, as well as cardiac epithelialization. Another possible interpretation for the temporal difference in N-cadherin antibody treatment is that there emerges a craniocaudal difference in the requirement for N-cadherin. It is likely that during heart development β-catenin may serve both as an adhesion-related molecule and as a signaling molecule involved in differentiation. The latter has been shown for other developing systems (Funayama *et al.*, 1995; Heasman *et al.*, 1994; McCrea *et al.*, 1993; Behrens *et al.*, 1996; Molenaar *et al.*, 1996).

The importance of cadherins to the process of epithelialization has been shown in earlier studies (McNeil *et al.*, 1990). Members of three large superfamilies of adhesion molecules are present in the cardiogenic regions during epithelialization, but all show different patterns of localization suggesting differing roles. Neither N-CAM, shown here, nor integrins (Linask, 1992a; as determined by the localization of β1 subunit) have the same pattern of expression as N-cadherin within the bilateral mesoderm preceding and during coelom development and epithelialization. Integrin is localized to the basal aspect of the cardiac mesoderm at sites of cell–substratum interactions and probably is important in anchoring the cardiac cells to the basal extracellular matrix and possibly also in cell signaling (Linask and Lash, 1986, 1990). N-CAM localizes ubiquitously within cells of the mesoderm and other germ layers and shows no specific changes in localization preceding the epithelialization step. As the cardiomycytes form an epithelium, N-CAM then becomes localized to the lateral sides to coincide with Na/K-ATPase expression (Linask, 1992a). N-CAM is not localized specifically to the apical lateral cell junctions where N-cadherin and the catenins localize. However, the immunostaining pattern of three different types of cell adhesion molecules suggests that the cardiomyocytes become ''locked-in place'' during the compartmentalization process and are involved in maintaining the compartment. It is very likely that cross-talk during signaling from these adhesion systems may be a factor in differentiation.

It appears that in cardiac myogenesis, as in skeletal myogenesis, a community of cells must be established in order for phenotypic differentiation to proceed efficiently (Gurdon *et al.*, 1993). Although the necessary regulatory genes may be present, they cannot alone support normal phenotypic differentiation without cadherin/β-catenin-mediated cell–cell interactions also being established. At present N-cadherin is the only cadherin molecule known to be expressed during these early stages in the heart field. However, additional cadherins may be identified. Other adhesion molecules as N-CAM and integrins also are present and may participate in maintaining the compartment. Since the submission of this article for review, a paper describing a N-cadherin null mutant is in press (Radice *et al.*, 1997). This paper presents similar results on the relationship between N-cadherin and abnormalities of the neural tube, somites, and heart. The embryonic lethality of the null mutants may relate to abnormal cardiac myogenesis and heart development, as seen by a thin and only weakly contracting heart wall. At a time when heart activity in the mouse must accommodate the extraembryonic circulation (see Gui *et al.*, 1996), the embryos die, i.e., on Days 9–9.5 of gestation. Recognizable hearts do form, however, and may reflect the participation of multiple adhesion molecules in cardiogenesis. Compensation may occur in the null mouse in the face of a chronic loss of N-cadherin. In our N-cadherin antibody treatment of chick embryos, an acute loss of N-cadherin occurs within specific windows of heart development. Compensatory pathways may not have the time to be established to normalize development. As a result, somewhat more striking perturbations occur. The formation of a three-dimensional heart tube and myogenic differentiation appear to involve independently regulated, but parallel, pathways (Linask and Gui, 1995). These parallel pathways may be coordinated by the expression of cell adhesion molecules.

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